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(54) Title: PEPTIDES THAT BIND TO THE HEPARIN BINDING DOMAIN OF VEGF AND VEGFR-2

(57) Abstract: The present invention relates to new peptides for targeting that bind to the heparin binding domain of vascular endothelial growth factor and its receptor vascular endothelial growth factor receptor 2, VEGFR-2. The invention further relates to their use in therapeutically effective treatment as well as for diagnostic imaging techniques.

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Field of invention

The present invention relates to new peptide-based compounds and their use in therapeutically effective treatment as well as for diagnostic imaging techniques. More specifically the invention relates to the use of such peptide-based compounds as targeting agents that bind to the heparin binding domain of vascular endothelial growth factor and its receptor vascular endothelial growth factor receptor 2 (VEGFR2/KDR(kinase insert domain-containing receptor)/flk-1 (fetal liver kinase)). VEGFR-2 is expressed on angiogenic endothelial cells, haematopoietic stem cells, endothelial precursor cells in the bone marrow, and several malignant cells. Contrast agents based on these peptides may thus be used for diagnosis of for example malignant diseases, heart diseases, endometriosis, inflammation-related diseases and rheumatoid arthritis. Moreover such agents may be used in therapeutic treatment of these diseases through inhibition of angiogenesis.

Background of invention

New blood vessels can be formed by two different mechanisms: angiogenesis or vasculogenesis. Angiogenesis is the formation of new blood vessels by sprouting/branching from existing vessels. The primary stimulus for this process may be inadequate supply of nutrients and oxygen (hypoxia) to cells in a tissue. The cells may respond by secreting angiogenic factors, of which there is many; one example, which is frequently referred to, is vascular endothelial growth factor (VEGF). These factors initiate the secretion of proteolytic enzymes that break down the proteins of the basement membrane, as well as inhibitors that limit the action of these potentially harmful enzymes. The other prominent effect of angiogenic factors is to cause endothelial cells to migrate and divide. Endothelial cells that are attached to the basement membrane, which forms a continuous sheet around blood vessels on the contraluminal side, do not undergo mitosis. The combined effect of loss of attachment and signals from the receptors for angiogenic factors is to cause the endothelial cells to move, multiply, and rearrange themselves, and finally to synthesise a basement membrane around the new vessels. Vasculogenesis is the generation of new vessels by recruiting endothelial precursor cells from the bone marrow. Newly published data shows that vasculogenesis not only is restricted to fetal blood vessel formation, but also occurs in the adult as response to various conditions. The bone marrow derived endothelial precursor cells recruited are also expressing VEGFR2.

Angiogenesis is prominent in the growth and remodelling of tissues, including wound healing and inflammatory processes. Tumours must initiate angiogenesis when they reach millimetre size in order to keep up their rate of growth. Angiogenesis is accompanied by characteristic changes in the endothelial cells and in their environment. The surface of these cells is remodelled in preparation for migration, and cryptic structures are exposed where the basement membrane is degraded, in addition to the variety of proteins which are involved in effecting and controlling proteolysis. In the case of tumours, the resulting network of blood vessels is usually disorganised, with the formation of sharp kinks and also arteriovenous shunts. Various glycosaminoglycans (GAGs) including heparan sulphate are important players in the angiogenic interactions. Several growth factors and their receptors including VEGF and VEGFR-2 have binding sites for heparan sulphate. Peptide mimics of GAGs may have important functions both as angiogenesis specific imaging agents, and as potential therapeutical agents through inhibition of angiogenesis.

Inhibition of angiogenesis is considered to be a promising strategy for anti tumour therapy. The transformations accompanying angiogenesis are also very promising as targets for diagnosis. An obvious example is malignant disease, but the concept also shows great promise in inflammation and a variety of inflammation-related diseases, including atherosclerosis. The macrophages of early atherosclerotic lesions are potential sources of angiogenic factors. These factors are also involved in re-vascularisation of infarcts in the myocardium.

Further examples of undesired conditions that are associated with neovascularization or angiogenesis implying the development or proliferation of new blood vessels, are shown below. Reference is also made in this regard to WO 98/47541.

Diseases and indications associated with angiogenesis are e.g. different forms of cancer and metastasis, e.g. breast, skin, colorectal, pancreatic, prostate, lung or ovarian cancer.

Other diseases and indications are inflammation (e.g. chronic), atherosclerosis, rheumatoid arthritis and gingivitis.

Further diseases and indications associated with angiogenesis are arteriovenous malformations, astrocytomas, choriocarcinomas, glioblastomas, gliomas, hemangiomas (childhood, capillary), hepatomas, hyperplastic endometrium, ischemic

myocardium, Kaposi sarcoma, macular degeneration, melanoma, neuroblastomas, occluding peripheral artery disease, osteoarthritis, psoriasis, retinopathy (diabetic, proliferative), scleroderma, seminomas and ulcerative colitis.

The malignant cells and the stroma cells up-regulate proteins that are involved in the process of angiogenesis. More or less specific markers are expressed on the endothelial cells. These markers include growth factor receptors such as VEGFR2. Immunohistochemical studies in combination with electron microscopy have demonstrated that VEGFR2 is expressed on the abluminal and luminal plasma membranes of vascular endothelial cells (Dvorak & Feng, 2001 J Histochem Cytochem, 49:419). VEGF produced by hypoxic tumour cells or stromal cells binds to the VEGFR2 on endothelial cells and stimulate angiogenesis. As complexes of VEGF and VEGFR2 are found predominantly on the abluminal side of the vascular endothelium, VEGFR2 available for targeting by circulating ligands is available at the luminal surface.

The present invention

It has now been found a new peptide targeting the heparin binding domain of vascular endothelial growth factor and its receptor vascular endothelial growth factor receptor 2, VEGFR 2. This new peptide can be used as a therapeutic agent in a pharmaceutical formulation by inhibiting the angiogenesis in the diseased area/tissue.

Further the peptide can be coupled to a known therapeutic agent that will be carried to the diseased area/tissue by the targeting abilities of the new peptide.

One or more peptide can further be coupled to a chelating agent or a reporter moiety either by direct bonding or via a linker moiety to act as a diagnostic imaging agent or a therapeutic active agent.

Detailed description of the invention

The invention is described in the patent claims. Specific features of the invention are outlined in the following detailed description and the Examples.

The three letter abbreviations used for the amino acids have the following meaning:

Arg - Arginine

Asp - Aspartic acid

Cys - Cysteine

Hcy	- Homocysteine
Gly	- Glycine
Val	- Valine
Tyr	- Tyrosine
Ile	- Isoleucine
Ser	- Serine
Leu	- Leucine
Asn	- Asparagine
Gln	- Glutamine
Ala	- Alanine
Met	- Methionine
Glu	- Glutamic acid
His	- Histidine
Thr	- Threonine
Phe	- Phenylalanine
Trp	- Tryptophan
Ser	- Serine

In a first aspect, the present invention provides a new peptide that targets the heparin binding domain of VEGF and its receptor VEGFR 2.

A peptide comprising the amino acid sequence of formula (I)

$Z^1-X^1-X^2-X^3-X^4-X^5-\text{Gly}-X^7-X^8-X^9-Z^2-Y^1$ (I)

wherein

X^1 is an amino acid selected from the group Ser, His, Thr, Ala, Gln, Phe, Gly and Ile

X^2 is an amino acid selected from the group Tyr, Arg and Phe

X^3 is an amino acid selected from the group Tyr, Ser, Asn, Glu, Asp and Thr

X^4 is an amino acid selected from the group Ser, Ala, Gly, Asp and Phe

X^5 is an amino acid selected from the group Asp and Ser,

X^7 is an amino acid selected from the group Thr, Val, Met, Ser, Trp, Tyr, Leu and Ala

X^8 is an amino acid selected from the group Tyr, Phe and Leu

X^9 is an amino acid selected from the group Asp, Ser and Glu

Z^1 represent an amino acid residue capable of forming a disulphide bond, preferably a cysteine or a homocysteine residue, or a residue capable of forming a thioether preferably the residue is $Q-C(=O)$ wherein Q represents $-(CH_2)_n$ or $-(CH_2)_n-C_6H_4$ where n represents a positive integer 1 to 10 or is absent and

Z^2 represent an amino acid residue capable of forming a disulphide bond, preferably a cysteine or a homocysteine residue or is absent

Y^1 represents 1-10 amino acids or is absent

or pharmaceutically acceptable salts thereof.

More specific the new peptide comprises the amino acid sequence of formula (II)

Z^1 - X^1 -Tyr- X^3 (-Ala/Ser)-Asp-Gly- X^7 -(Tyr/Phe)-Asp- Z^2 - Y^1 (II)

wherein

X^1 is an amino acid selected from the group Ser, His, Thr, Ala, Gln, Phe, Gly and Ile

X^3 is an amino acid selected from the group Tyr, Ser, Asn, Glu, Asp and Thr

X^7 is an amino acid selected from the group Thr, Val, Met, Ser, Trp, Tyr, Leu and Ala

Z^1 represent an amino acid residue capable of forming a disulphide bond, preferably a cysteine or a homocysteine residue, or a residue capable of forming a thioether preferably the residue is Q-C(=O) wherein Q represents $-(CH_2)_n$ or $-(CH_2)_n-C_6H_4$ where n represents a positive integer 1 to 10 or is absent and

Z^2 represent an amino acid residue capable of forming a disulphide bond, preferably a cysteine or a homocysteine residue or is absent

Y^1 represents 1-10 amino acids or is absent

or pharmaceutically acceptable salts thereof.

Further preferred are peptides comprising an amino acid sequence as follow:

Cys-Ser-Tyr-Tyr-Ser-Asp-Gly-Val-Tyr-Asp-Cys, (SEQ ID NO 1),

Cys-His-Tyr-Ser-Ser-Asp-Gly-Thr-Tyr-Asp-Cys, (SEQ ID NO 2),

Cys-Thr-Tyr-Asn-Gly-Asp-Gly-Ser-Phe-Asp-Cys, (SEQ ID NO 3),

Cys-Ala-Tyr-Glu-Ala-Asp-Gly-Trp-Phe-Asp-Cys, (SEQ ID NO 4),

Cys-Ser-Tyr-Ser-Ala-Asp-Gly-Thr-Leu-Asp-Cys, (SEQ ID NO 5),

Cys-Gln-Tyr-Asp-Ser-Ser-Gly-Met-Tyr-Asp-Cys, (SEQ ID NO 6),

Cys-Phe-Phe-Asp-Ser-Ser-Gly-Tyr-Phe-Asp-Cys, (SEQ ID NO 7),

Cys-Thr-Tyr-Ser-Ala-Asp-Gly-Leu-Tyr-Asp-Cys, (SEQ ID NO 8),

Cys-His-Phe-Asp-Gly-Asp-Gly-Ser-Tyr-Asp-Cys, (SEQ ID NO 9),

Cys-Thr-Tyr-Glu-Pro-Ser-Gly-Met-Tyr-Asp-Cys, (SEQ ID NO 10),

Cys-Gln-Tyr-Thr-Ala-Asp-Gly-Ala-Phe-Asp-Cys, (SEQ ID NO 11),

Cys-Ile-Tyr-Glu-Ser-Asp-Gly-Met-Phe-Ser-Cys, (SEQ ID NO 12),

Cys-Gly-Arg-Ser-Asp-Gly-Thr-Trp-Tyr-Glu-Cys, (SEQ ID NO 13) or

Cys-Ser-Tyr-Tyr-Ala-Asp-Gly-Met-Tyr-Ser-Cys, (SEQ ID NO 14).

Viewed from another aspect the invention provides new peptide-based compounds as defined by formula (III).

V-L-Z Formula (III)

wherein the vector V is a peptide as defined above, L represents a bond, a spacer or linker and Z represents an antineoplastic agent, a reporter moiety or a group that optionally can carry an imaging moiety M.

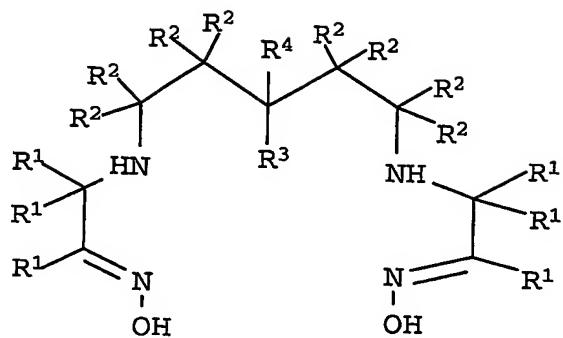
The role of the linker L is to couple vector to reporter, and in the case where L is a spacer moiety the role of L is to distance the relatively bulky chelating agent from the active site of the peptide component. The spacer moiety L is also applicable to distance a bulky antineoplastic agent from the active site of the peptide.

A linker moiety may serve to link one vector to one reporter; alternatively it may link together more than one vector and/or more than one reporter. Likewise a reporter or a vector may be linked to more than one linker. Use in this way of a plurality of reporters (e.g. several linker-reporter moieties attached to one vector or several reporters attached to one linker itself attached to one vector) may enable the detectability of the contrast agent to be increased (e.g. by increasing its radioopacity, echogenicity or relaxivity) or may enable it to be detected in more than one imaging modality. Use in this way of a plurality of vectors may e.g. increase the targeting efficiency of a contrast agent or may make the contrast agent/therapeutic agent able to target more than one site, e.g. different receptors for an agent which has receptor heterogeneity.

The linker moiety L may be a simple bond or may be represented by other linkers well known in the art, e.g. as described in WO 01/77145 pages 23-27, the content of which are incorporated herein by reference.

Z can be represented by stabilised gas-filled microbubbles. In this aspect of the invention the compounds of formula (III) can be used for targeted ultrasound imaging. Each of the microbubbles may carry several vectors V.

Z can further be represented by a chelating agent of Formula IV:

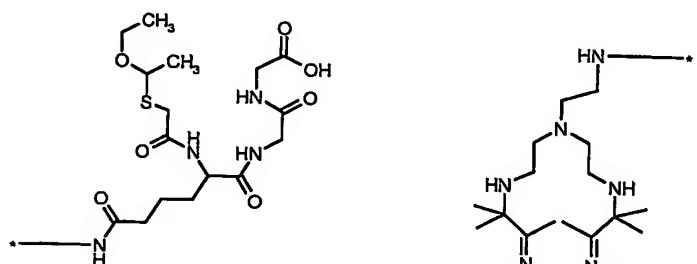


(IV)

where:

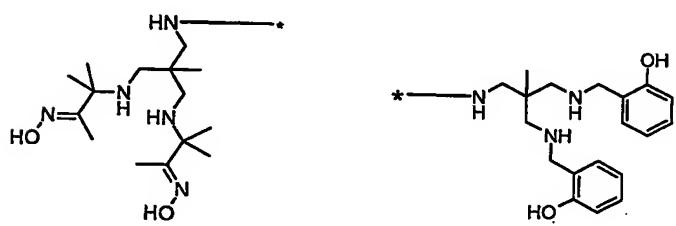
each R^1 , R^2 , R^3 and R^4 is independently an R group;

each R group is independently H or C_{1-10} alkyl, C_{3-10} alkylaryl, C_{2-10} alkoxyalkyl, C_{1-10} hydroxyalkyl, C_{1-10} alkylamine, C_{1-10} fluoroalkyl, or 2 or more R groups, together with the atoms to which they are attached form a carbocyclic, heterocyclic, saturated or unsaturated ring, or can represent a chelating agent given by formulas a, b, c and d.



a.

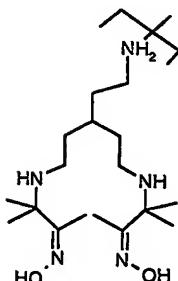
b



c

d

A preferred example of a chelating agent is represented by formula e.



e

Conjugates comprising chelating agents of Formula (IV) can be radiolabelled to give good radiochemical purity, RCP, at room temperature, under aqueous conditions at near neutral pH. The risk of opening the disulphide bridge of the peptide component at room temperature is less than at an elevated temperature. A further advantage of radiolabelling the conjugates at room temperature is a simplified procedure in a hospital pharmacy.

However the compounds defined in Formula (III) may also comprise chelating agents, Z, as defined in WO 01/77145, Table I, pages 11-15.

In some aspects of the invention, Z comprises a reporter moiety, M, where said reporter moiety comprises a radionuclide. Further definitions of chelating agents are listed in WO 01/77145, Table I, pages 11-15, the content of which are incorporated herein by reference.

In one aspect of the present invention of formula (III) Z is represented by an antineoplastic agent. In this aspect the compound will target an angiogenic site associated with cancer and bring the antineoplastic agent to the diseased area. The antineoplastic agent may be represented by cyclophosphamide, chloroambucil, busulphan, methotrexate, cytarabine, fluorouracil, vinblastine, paclitaxel, doxorubicin, daunorubicin, etoposide, teniposide, cisplatin, amsacrine, docetaxel, but a wide range of other antineoplastic agents may also be used.

The peptide component of compounds of formula (III) may be in a cyclic configuration, i.e. by a disulphide bond or it may be linear.

The peptide component of the conjugates described herein have preferably no free amino- or carboxy-termini. This introduces into these compounds a significant increase in resistance against enzymatic degradation and as a result they have an increased in vivo stability as compared to many known free peptides.

The reporter moieties (Z) in the contrast agents of the invention may be any moiety capable of detection either directly or indirectly in an in vivo diagnostic imaging procedure.

For MR imaging the reporter will either be a non zero nuclear spin isotope (such as ^{18}F) or a material having unpaired electron spins and hence paramagnetic, superparamagnetic, ferrimagnetic or ferromagnetic properties; for light imaging the reporter will be a light scatterer (e.g. a coloured or uncoloured particle), a light absorber or a light emitter; for magnetometric imaging the reporter will have detectable magnetic properties; for electrical impedance imaging the reporter will affect electrical impedance; and for scintigraphy, SPECT, PET, and the like, the reporter will be a radionuclide.

Stated generally, the reporter may be (1) a chelatable metal or polyatomic metal-containing ion (i.e. TcO , etc), where the metal is a high atomic number metal (e.g. atomic number greater than 37), a paramagnetic species (e.g. a transition metal or lanthanide), or a radioactive isotope, (2) a covalently bound non-metal species which is an unpaired electron site (e.g. an oxygen or carbon in a persistent free radical), a high atomic number non-metal, or a radioisotope, (3) a polyatomic cluster or crystal containing high atomic number atoms, displaying cooperative magnetic behaviour (e.g. superparamagnetism, ferrimagnetism or ferromagnetism) or containing radionuclides.

Examples of particular preferred reporter groups (M) are described in more detail below.

Chelated metal reporters are preferably chosen from the group below; ^{90}Y , $^{99\text{m}}\text{Tc}$, ^{111}In , ^{47}Sc , ^{67}Ga , ^{51}Cr , $^{177\text{m}}\text{Sn}$, ^{67}Cu , ^{167}Tm , ^{97}Ru , ^{188}Re , ^{177}Lu , ^{199}Au , ^{203}Pb and ^{141}Ce .

The metal ions are desirably chelated by chelating agents on the linker moiety. Further examples of suitable chelating agents are disclosed in US-A-4647447, WO89/00557, US-A-5367080, US-A-5364613.

Methods for metallating any chelating agents present are within the level of skill in the art. Metals can be incorporated into a chelant moiety by any one of three general methods: direct incorporation, template synthesis and/or transmetallation. Direct incorporation is preferred.

Thus it is desirable that the metal ion be easily complexed to the chelating agent, for example, by merely exposing or mixing an aqueous solution of the chelating agent-containing moiety with a metal salt in an aqueous solution preferably having a pH in the range of about 4 to about 11. The salt can be any salt, but preferably the salt is a water soluble salt of the metal such as a halogen salt, and more preferably such salts are selected so as not to interfere with the binding of the metal ion with the chelating agent. The chelating agent-containing moiety is preferably in aqueous solution at a pH of between about 5 and about 9, more preferably between pH about 6 to about 8. The chelating agent-containing moiety can be mixed with buffer salts such as citrate, carbonate, acetate, phosphate and borate to produce the optimum pH. Preferably, the buffer salts are selected so as not to interfere with the subsequent binding of the metal ion to the chelating agent.

The following isotopes or isotope pairs can be used for both imaging and therapy without having to change the radio-labelling methodology or chelator: $^{47}\text{Sc}_{21}$; $^{141}\text{Ce}_{58}$; $^{188}\text{Re}_{75}$; $^{177}\text{Lu}_{71}$; $^{199}\text{Au}_{79}$; $^{47}\text{Sc}_{21}$; $^{131}\text{I}_{53}$; $^{67}\text{Cu}_{29}$; $^{131}\text{I}_{53}$ and $^{123}\text{I}_{53}$; $^{188}\text{Re}_{75}$ and $^{99\text{m}}\text{Tc}_{43}$; $^{80}\text{Y}_{39}$ and $^{87}\text{Y}_{39}$; $^{47}\text{Sc}_{21}$ and $^{44}\text{Sc}_{21}$; $^{90}\text{Y}_{39}$ and $^{123}\text{I}_{53}$; $^{146}\text{Sm}_{62}$ and $^{153}\text{Sm}_{62}$; and $^{90}\text{Y}_{39}$ and $^{111}\text{In}_{49}$.

Preferred non-metal atomic reporters include radioisotopes such as ^{123}I , ^{131}I and ^{18}F as well as non zero nuclear spin atoms such as ^{19}F , and heavy atoms such as I.

In a further embodiment of this invention, the use of radioisotopes of iodine or fluorine is specifically contemplated. For example, if the peptide or linker is comprised of substituents that can be chemically substituted by iodine or fluorine in a covalent bond forming reaction, such as, for example, substituents containing hydroxyphenyl or p-nitrobenzoyl functionality, such substituents can be labeled by methods well known in the art with a radioisotope of iodine or fluorine respectively. These species can be used in therapeutic and diagnostic imaging applications. While, at the same time, a metal attached to a chelating agent on the same peptide-linker can also be used in either therapeutic or diagnostic imaging applications.

The compounds of formula (III) may be therapeutically effective in the treatment of disease states as well as detectable in *in vivo* imaging. Thus for example the vector on the reporter moieties may have therapeutic efficacy, e.g. by virtue of the radiotherapeutic effect of a radionuclide reporter of the vector moiety.

The present invention also provides a pharmaceutical composition comprising an effective amount (e.g. an amount effective for enhancing image contrast in *in vivo* imaging) of a compound of general formula (III) or a salt thereof, together with one or more pharmaceutically acceptable adjuvants, excipients or diluents.

The invention further provides a pharmaceutical composition for treatment of a disease comprising an effective amount of a compound of general formula (III), or a salt thereof, together with one or more pharmaceutically acceptable adjuvants, excipients or diluents.

Use of the new peptide and the compounds of formula (III) in the manufacture of therapeutic compositions (medicament) and in methods of therapeutic or prophylactic treatment, preferably treatment of cancer, of the human or animal body are thus considered to represent further aspects of the invention.

Viewed from a further aspect the invention provides the use of a compound of formula (III) for the manufacture of a contrast medium for use in a method of diagnosis involving administration of said contrast medium to a human or animal body and generation of an image of at least part of said body.

Viewed from a still further aspect the invention provides a method of generating enhanced images of a human or animal body previously administered with a contrast agent composition comprising a compound as defined by formula (III), which method comprises generating an image of at least part of said body.

Viewed from a further aspect the invention provides a method of monitoring the effect of treatment of a human or animal body with a drug to combat a condition associated with cancer, preferably angiogenesis, e.g. a cytotoxic agent, said method involving administering to said body an agent of formula (III) and detecting the uptake of said agent by cell receptors, preferably endothelial cell receptors and in particular VEGF

receptors, said administration and detection optionally but preferably being effected repeatedly, e.g. before, during and after treatment with said drug.

These new compounds may be used in therapeutically effective treatments as well as for imaging purposes. Further these new compounds may be used for drug delivery purposes.

General procedures

The abbreviations used have the following meanings:

Fmoc	- 9-fluorenylmethoxycarbonyl
Acm	- acetamidomethyl
TFA	- trifluoroacetic acid
ACN	- acetonitril
RP-HPLC	- reversed phase high pressure liquid chromatography
DMF	- dimethylformamide
NMM	- N-methylmorpholine
DCM	- dichloromethane
NMP	- N-methylpyrrolidone
HBTU	-2-(1H-benzotriazol-1-yl)-1,1,3,3-tetramethyluronium-hexafluorophosphate
HOEt	- 1-hydroxy-benzotriazole
DIEA	- diisopropylethylamine
DTT	- dithiotreitol

The peptides of the present invention can be synthesised using all the known methods of chemical synthesis but particularly useful is the solid-phase methodology of Merrifield employing an automated peptide synthesiser (J. Am. Chem. Soc., 85: 2149 (1964)). Typically, the desired sequences are assembled by solid-phase peptide synthesis. Standard procedures for the synthesis strategy employed for the examples of this invention are described in E. Atherton & R.C. Sheppard, "Solid phase peptide synthesis: a practical approach, 1989, IRL Press, Oxford.

For example, a synthesis resin with an acid-labile linker group, to which the desired protected C-terminal amino acid residue is attached by amide bond formation, is used. In the following examples 1 and 2, a so-called Rink amide AM resin with a (dimethoxyphenyl-aminomethyl)-phenoxy-derived linker was applied (Rink, H. *Tetrahedron Lett.* (1987), 30, 3787). Moreover, in example 3 an even more acid

labile resin was used, a so-called XAL-MBHA resin with a xanthenyl-derived linker (Han, Y.; Bontems, S. L.; Hegyes, P.; Munson, M. C.; Minor, C. A.; Kates, S. A.; Albericio, F.; Barany, G., *J. Org. Chem.* (1996), 61, 6326-6339).

The N^α-amino-protecting group is then removed and the second amino acid in the sequence is coupled using suitable condensation reagents. N^α-amino-deprotection and coupling cycles are then repeated in alternating steps until the sequence of interest is assembled. Amino acids with a temporary N^α-amino protecting group and permanent protecting groups for the functional side chains are employed. Generally, all reactive groups present (for example amino, hydroxyl, thiol and carboxyl groups) will be protected during peptide synthesis.

A wide range of protecting groups for amino acids is known (see, e.g., Greene, T.W. & Wuts, P.G.M. (1991) *Protective groups in organic synthesis*, John Wiley & Sons, New York). Thus, examples of amino protecting groups which may be employed include 9-fluorenylmethoxycarbonyl (Fmoc), benzyloxycarbonyl, t-butyloxycarbonyl, etc. When the peptide is built up from the C-terminal end, the N^α-amino-protecting group will need to be removed selectively prior to the next coupling step. One particularly useful group for such temporary amine protection is the Fmoc group which can be removed selectively by treatment with piperidine in an organic solvent. Carboxyl protecting groups which may be employed include for example t-butyl, benzyl, trityl, etc. The thiol protecting group used can be semi-permanent for example the *p*-methoxytrityl group or permanent including the trityl and acetamidomethyl groups. It will be appreciated that a wide range of other such groups are known in the art.

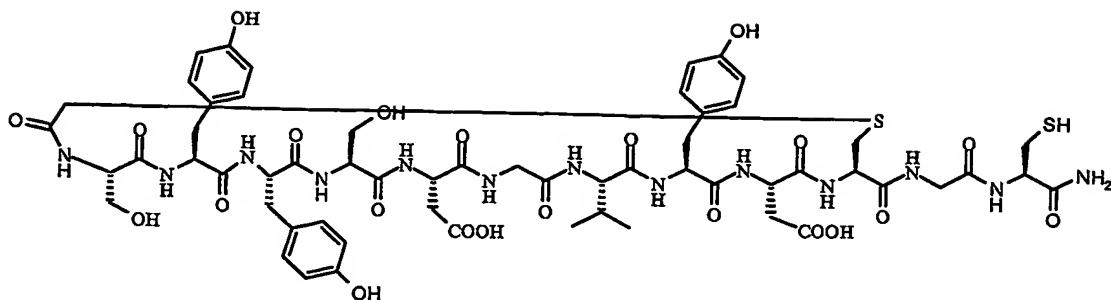
Finally the peptide is cleaved from the synthesis resin and the permanent side-chain protecting groups are removed, usually simultaneously as in examples 1 and 2 below. Alternatively, a synthesis resin is used which allows detachment of the peptide under mild conditions where the side-chain protecting groups are stable, affording protected peptides, as in example 3 below.

The invention is further illustrated by the following none limiting examples.

Examples

Example 1

cyclo-[CH₂CO-Ser-Tyr-Tyr-Ser-Asp-Gly-Val-Tyr-Asp-Cys]-Gly-Cys-NH₂



The peptidyl resin corresponding to the above sequence was assembled on a Rink Amide AM resin (0.74 mmol/g; from NovaBiochem) using an Applied Biosystems (Perkin Elmer) model 433A peptide synthesizer. Fmoc deprotection was achieved with conductivity monitoring using 20% piperidine in N-methylpyrrolidone (NMP). The washing solvent was NMP. The residues (from the carboxyl terminus) were assembled on a 0.25 mmol scale using single couplings with a 4-fold molar excess of Fmoc-amino acids (1 mmol cartridges) and 2-(1H-benzotriazol-1-yl)-1,1,3,3-tetramethyluronium-hexafluorophosphate (HBTU)/1-hydroxy-benzotriazole (HOBr)/diisopropylethylamine (DIEA) in NMP using 2.5 hours coupling cycles. The amino acid-side chain protecting groups used were *tert*-butyl for Asp, Ser and Tyr, trityl for Cys¹⁰ and acetamidomethyl (AcM) for Cys¹². The Gly residue coupled in position 6 was used with an additional N^a-protecting group (2-Fmoc-oxy-4-methoxybenzyl) in order to suppress aspartimide formation (Packman, L.C. 1995, *Tetrahedron Lett.* 36, p.7523-7526).

The assembled peptidyl resin was then transferred to a manual nitrogen bubbler apparatus (Wellings, D.A., Atherton, E. (1997) in Methods in Enzymology (Fields, G. ed), 289, p. 53-54, Academic Press, New York). The N-terminus was Fmoc-deprotected and then bromoacetylated using a 10-fold molar excess of bromoacetyl bromide and N-methylmorpholine (NMM) in dimethylformamide (DMF) for 1 hour. The completed peptidyl resin was washed with DMF and dichloromethane (DCM) and dried *in vacuo*.

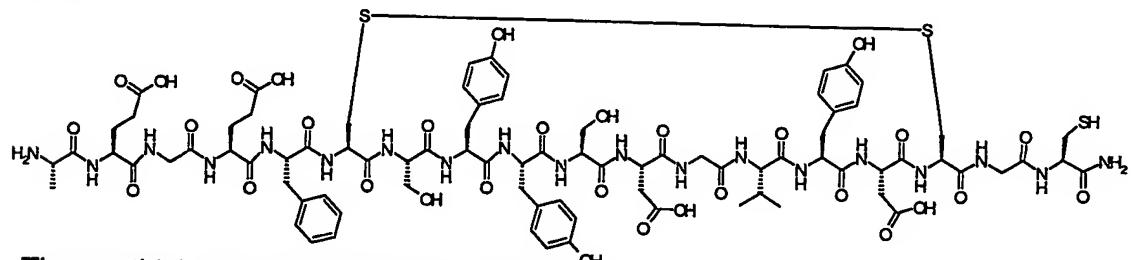
Simultaneous cleavage of the peptide from the resin and removal of the side-chain protecting groups (except the acetamidomethyl group) from the peptide, was effected by treating an aliquot of the peptidyl resin (0.1 mmol) with trifluoroacetic acid (TFA) containing 2.5% triisopropylsilane and 2.5% water for 2 hours. The resin residue was filtered off and washed with small quantities of neat TFA. The combined filtrate and

washings were concentrated by rotary evaporation and then triturated with diethyl ether to obtain the crude peptide. The precipitate was isolated by centrifugation, washed with ether and then lyophilized from 50% acetonitrile (ACN)-0.1% aq TFA, giving a crude product yield of 150 mg.

The linear crude peptide was cyclized by thioetherbridge formation, effected by stirring the peptide in 300 ml 50% ACN-water at pH 8 (adjusted by liquid ammonia) for 30 min at RT. The cyclized product was isolated by lyophilization. The Acm-protecting group was then removed by treating the peptide with a 5-fold molar excess of mercury(II)-acetate at pH 4-4.5 (adjusted with acetic acid) in 25% ACN-water. After stirring for 1 h at RT dithiotreitol (DTT) was added as a solid to the mixture in a 4-fold molar excess with respect to mercury(II)-acetate. The reaction was stirred for 2.5 h at RT and the precipitate that formed was removed by centrifugation. The supernatant was lyophilized to give the fully deprotected cyclized crude peptide. An aliquot (0.074 mmol) of the crude product was dissolved in 10% ACN-0.1% aq. TFA (50 ml) filtered and purified by preparative RP-HPLC. The column (Phenomenex Luna C18 10 μ , 250 x 50 mm) was eluted at 50 ml/ min with a gradient of 10 to 25% ACN in 0.1% aq TFA over 60 min. The desired peak fractions were pooled and lyophilized to afford 21 mg of pure peptide. Analytical RP-HPLC: t_R = 17.2 min, purity 99% (Phenomenex Luna 5 μ , 4.6 x 250 mm, 0-40 % ACN in 0.1% aq TFA over 20 min at 1 ml/min, λ =215 nm). Electrospray MS: [M+H]⁺ of product expected at 1370.5 m/z, found at 1370.3 m/z.

Example 2

Disulfide-Ala-Glu-Gly-Glu-Phe-Cys-Ser-Tyr-Tyr-Ser-Asp-Gly-Val-Tyr-Asp-Cys-Gly-Cys-NH₂

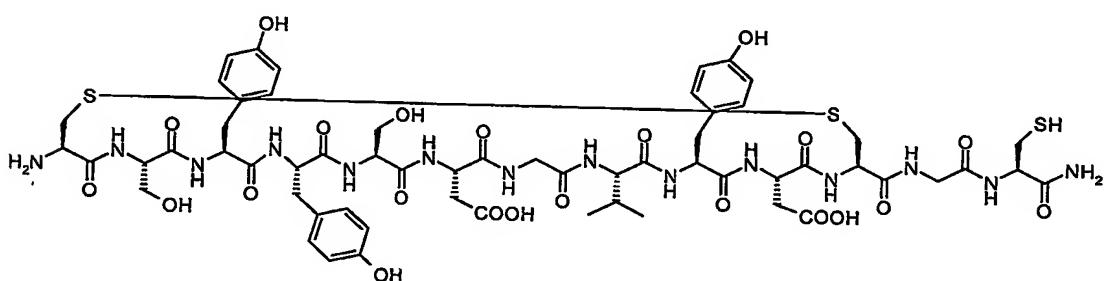


The peptidyl resin corresponding to the above sequence was assembled on a Fmoc-XAL-MBHA resin (0.47 mmol/g; from Bachem) in a similar fashion to the corresponding peptidyl resin of Example 1. Selective removal of the peptide from the resin while maintaining the side-chain protecting groups, was carried out on a 0.085 mmol scale using the procedure described in Example 1.

The reduced protected peptide was oxidized by re-dissolving the crude peptide in DMF (25 ml) and adding the solution to 65% acetonitrile (ACN)-H₂O (750 ml). It was difficult to obtain a homogeneous solution so more ACN (250 ml), DMF (100ml) and DMSO (200 ml) was added in order to obtain a close to homogeneous solution. The pH was adjusted to ~7.9 with dilute liq. NH₃ and the oxidation was let proceed for 48 h. At this point the solution was concentrated down by rotary evaporation.

Example 3

Cys¹⁻¹¹; Cys-Ser-Tyr-Tyr-Ser-Asp-Gly-Val-Tyr-Asp-Cys-Gly-Cys-NH₂



The peptidyl resin corresponding to the above sequence was assembled on a Fmoc-XAL-MBHA resin (0.47 mmol/g; from Bachem) in a similar fashion to the corresponding peptidyl resin of Example 1. The side-chain protecting groups for the Cys residues used here were *p*-methoxytrityl for Cys^{1,11} and trityl for Cys¹³.

An aliquot of the assembled peptidyl resin (0.035 mmol) was transferred to a manual nitrogen bubbler and treated with DCM containing 5% TIS and 1% TFA for 5 x 5 min in order to liberate the fully side-chain protected peptide from the resin. Care was taken to avoid evaporation of DCM and thus up-concentration of TFA. The filtrates were neutralized by collection in a flask containing pyridine (2 equivalents per equivalent TFA, 1 ml) and MeOH (1 ml / 10 ml cleavage solution, 5 ml). The combined filtrates were concentrated by rotary evaporation until precipitation occurred and the precipitate was isolated by centrifugation.

Cyclization of the protected peptide by disulfide formation was carried out by adding a solution of the peptide in DMF (10 ml) to 60% ACN-water (250 ml), giving a

concentration of <0.25 mg/ml. The pH was adjusted to ~8.6 with dilute liq. NH₃ and the oxidation was let proceed for 40 h. At this point the solution was concentrated down and the product was isolated by centrifugation.

An aliquot (~18 mg) of the crude product was dissolved in 1 ml DMF and 3 ml 65% ACN-0.1% aq TFA filtered and purified by preparative RP-HPLC. The column (Vydac 218 TP1022, 22 x 250 mm) was eluted at 10 ml/ min with a gradient of 65 to 100% ACN in 0.1% aq TFA over 40 min. The desired peak fractions were pooled and lyophilized. Analytical RP-HPLC: t_R = 10.3 min, purity 95% (Phenomenex Luna 3 μ , 2.0 x 50 mm, 50-100 % ACN in 0.1% aq TFA over 10 min at 0.4 ml/min, λ =215 nm). MALDI-MS: [M+Na]⁺ of product expected at 2225.8 m/z, found at 2230.9 m/z.

Final deprotection of the peptide was effected by acidolytic treatment for 1 hr as described in Example 1. Yield: 1.3 mg. Analytical RP-HPLC: t_R = 5.97 min, purity 65% (Phenomenex Luna 3 μ , 4.6 x 50 mm, 0-40 % ACN in 0.1% aq TFA over 10 min at 2 ml/min, λ =215 nm). ESI-MS: [M+H]⁺ of product expected at 1431.5 m/z, found at 1431.4 m/z. ESI-MS/MS: [M+H]⁺ of the *des*-Gly-Cys product expected at 1254.4 m/z, found at 1254.3 m/z. The peptide product was used as such without further purification.

Claims

1. A peptide comprising the amino acid sequence of formula (I)

$Z^1-X^1-X^2-X^3-X^4-X^5-\text{Gly}-X^7-X^8-X^9-Z^2-Y^1$ (I)

or formula (II)

$Z^1-X^1-\text{Tyr}-X^3(\text{-Ala/Ser})-\text{Asp}-\text{Gly}-X^7-(\text{Tyr/Phe})-\text{Asp}-Z^2-Y^1$ (II)

wherein

X^1 is an amino acid selected from the group Ser, His, Thr, Ala, Gln, Phe, Gly and Ile

X^2 is an amino acid selected from the group Tyr, Arg and Phe

X^3 is an amino acid selected from the group Tyr, Ser, Asn, Glu, Asp and Thr

X^4 is an amino acid selected from the group Ser, Ala, Gly, Asp and Phe

X^5 is an amino acid selected from the group Asp and Ser,

X^7 is an amino acid selected from the group Thr, Val, Met, Ser, Trp, Tyr, Leu and Ala

X^8 is an amino acid selected from the group Tyr, Phe and Leu

X^9 is an amino acid selected from the group Asp, Ser and Glu

Z^1 represent an amino acid residue capable of forming a disulphide bond, preferably a cysteine or a homocysteine residue, or a residue capable of forming a thioether preferably the residue is $Q-C(=O)$ wherein Q represents $-(CH_2)_n$ or $-(CH_2)_n-C_6H_4$ where n represents a positive integer 1 to 10 or is absent and

Z^2 represent an amino acid residue capable of forming a disulphide bond, preferably a cysteine or a homocysteine residue or is absent

Y^1 represents 1-10 amino acids or is absent

or pharmaceutically acceptable salts thereof.

2. A peptide according to claim 1 of the amino acid sequence

Cys-Ser-Tyr-Tyr-Ser-Asp-Gly-Val-Tyr-Asp-Cys, (SEQ ID NO 1),

Cys-His-Tyr-Ser-Ser-Asp-Gly-Thr-Tyr-Asp-Cys, (SEQ ID NO 2),

Cys-Thr-Tyr-Asn-Gly-Asp-Gly-Ser-Phe-Asp-Cys, (SEQ ID NO 3),

Cys-Ala-Tyr-Glu-Ala-Asp-Gly-Trp-Phe-Asp-Cys, (SEQ ID NO 4),

Cys-Ser-Tyr-Ser-Ala-Asp-Gly-Thr-Leu-Asp-Cys, (SEQ ID NO 5),

Cys-Gln-Tyr-Asp-Ser-Ser-Gly-Met-Tyr-Asp-Cys, (SEQ ID NO 6),

Cys-Phe-Phe-Asp-Ser-Ser-Gly-Tyr-Phe-Asp-Cys, (SEQ ID NO 7),

Cys-Thr-Tyr-Ser-Ala-Asp-Gly-Leu-Tyr-Asp-Cys, (SEQ ID NO 8),

Cys-His-Phe-Asp-Gly-Asp-Gly-Ser-Tyr-Asp-Cys, (SEQ ID NO 9),

Cys-Thr-Tyr-Glu-Pro-Ser-Gly-Met-Tyr-Asp-Cys, (SEQ ID NO 10),
 Cys-Gln-Tyr-Thr-Ala-Asp-Gly-Ala-Phe-Asp-Cys, (SEQ ID NO 11),
 Cys-Ile-Tyr-Glu-Ser-Asp-Gly-Met-Phe-Ser-Cys, (SEQ ID NO 12),
 Cys-Gly-Arg-Ser-Asp-Gly-Thr-Trp-Tyr-Glu-Cys, (SEQ ID NO 13) or
 Cys-Ser-Tyr-Tyr-Ala-Asp-Gly-Met-Tyr-Ser-Cys, (SEQ ID NO 14).

3. A targetable diagnostic and/ or therapeutically active agent of formula (III)

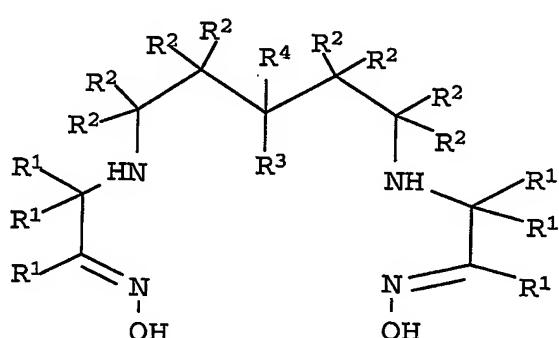
V-L-Z Formula (III)

wherein the vector V is a peptide according to claim 1- 2

L represents a bond, a spacer or a linker and

Z represents an antineoplastic agent, a reporter moiety or a group that optionally can carry an imaging moiety M.

4. An agent as claimed in claim 3 where Z is a chelating agent of Formula IV



(IV)

where:

each R¹, R², R³ and R⁴ is independently an R group;

each R group is independently H or C₁₋₁₀ alkyl, C₃₋₁₀ alkylaryl, C₂₋₁₀ alkoxyalkyl, C₁₋₁₀ hydroxyalkyl, C₁₋₁₀ alkylamine, C₁₋₁₀ fluoroalkyl, or 2 or more R groups, together with the atoms to which they are attached form a carbocyclic, heterocyclic, saturated or unsaturated ring.

5. An agent as claimed in any of the previous claims 3 to 4 wherein Z comprises a reporter moiety, M wherein the reporter moiety M comprises metal radionuclides,

paramagnetic metal ions, fluorescent metal ions, heavy metal ions or cluster ions.

6. An agent as claimed in claim 5 wherein the reporter moiety M comprises ⁹⁰Y, ^{99m}Tc, ¹¹¹In, ⁴⁷Sc, ⁶⁷Ga, ⁵¹Cr, ^{177m}Sn, ⁶⁷Cu, ¹⁶⁷Tm, ⁹⁷Ru, ¹⁸⁸Re, ¹⁷⁷Lu, ¹⁹⁹Au, ²⁰³Pb, ¹⁴¹Ce or ¹⁸F.

7. An agent as claimed in claims 3 to 6 where each reporter (Z) can carry a multiplicity of vectors V.

8. An agent as claimed in claim 3 where the antineoplastic agent Z represent cyclophosphamide, chloroambucil, busulphan, methotrexate, cytarabine, fluorouracil, vinblastine, paclitaxel, doxorubicin, daunorubicin, etoposide, teniposide, cisplatin, amsacrine or docetaxel.

9. A pharmaceutical composition comprising an effective amount of a compound of general Formula (III) or a salt thereof, together with one or more pharmaceutically acceptable adjuvants, excipients or diluents for use in enhancing image contrast in *in vivo* imaging or for treatment of a disease.

10. A method of generating enhanced images of a human or animal body previously administered with a contrast agent composition comprising a compound as claimed in claims 3 to 7, which method comprises generating an image of at least part of said body.

PN0273_Heparin_Sequence_listing

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PN0273_Heparin_Sequence_listing

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INTERNATIONAL SEARCH REPORT

International Application No

NO 03/00444

A. CLASSIFICATION OF SUBJECT MATTER

IPC 7 C07K2/00 C07K7/06 C07K7/08 A61K47/48 //C07K14/475,
C07K14/71

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 7 C07K A61K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

EP0-Internal, BIOSIS, WPI Data, PAJ, CHEM ABS Data

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 01 52875 A (LUDWIG INST CANCER RES) 26 July 2001 (2001-07-26) abstract page 19, line 10 -page 20, line 30 page 26, line 1 - line 19; examples ---	1-10
X	WO 99 40947 A (ESHIMA DENNIS ; POLLAK ALFRED (CA); THORNBACH JOHN (CA); FAUCONNIER) 19 August 1999 (1999-08-19) abstract page 12, line 2 - line 13; examples 2,6 claims ---	1-10

Further documents are listed in the continuation of box C.

Patent family members are listed in annex.

Special categories of cited documents :

- "A" document defining the general state of the art which is not considered to be of particular relevance
- "E" earlier document but published on or after the international filing date
- "L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
- "O" document referring to an oral disclosure, use, exhibition or other means
- "P" document published prior to the international filing date but later than the priority date claimed

- "T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
- "X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
- "Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.
- "&" document member of the same patent family

Date of the actual completion of the international search

5 May 2004

Date of mailing of the International search report

03 JUN 2004

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Authorized officer

TERESE PERSSON /EÖ

INTERNATIONAL SEARCH REPORT

International Application No

NO 03/00444

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT		
Category	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	<p>DATABASE HCPLUS [Online] MILLENNIUM PHARMACEUTICALS, INC.: "Humanized anti mammalian CC-chemokine receptor 2 (CCR2) antibodies and uses in therapeutics, prophylaxis and diagnosis" retrieved from STN INTERNATIONAL Database accession no. 2001:582061 XP002279014 see sequence in the end, aa 60-68 abstract -& WO 01 57226 A (MILLENIUM PHARMACEUTICALS, INC.) 9 August 2001 (2001-08-09) the whole document</p> <p>---</p> <p>WO 97 02479 A (UNIV YALE ;CAI XIAOHONG (US); GAREN ALAN (US)) 23 January 1997 (1997-01-23) sequence 48, aa 94-105</p> <p>---</p> <p>US 5 981 229 A (PEARCE BARBARA J ET AL) 9 November 1999 (1999-11-09) sequence 58, aa 127-135</p> <p>---</p> <p>WO 99 14321 A (INST MEDICAL W & E HALL ;PUTHALAKATH HAMSA (AU); REILLY LORRAINE O) 25 March 1999 (1999-03-25) sequence 6, aa 86-94</p> <p>---</p> <p>US 5 948 667 A (MOLONEY MAURICE M ET AL) 7 September 1999 (1999-09-07) sequence 2, aa 60-69</p> <p>---</p> <p>WO 99 10498 A (HELENTJARIS TIMOTHY G ;PIONEER HI BRED INT (US); WANG XUN (US); BO) 4 March 1999 (1999-03-04) sequence 11, aa 14-22</p> <p>---</p> <p>EL-SHEIKH AMR ET AL: "A novel vascular endothelial growth factor heparin-binding domain substructure binds to glycosaminoglycans in vivo and localizes to tumor microvascular endothelium." CANCER RESEARCH, vol. 62, no. 23, 1 December 2002 (2002-12-01), pages 7118-7123, XP002279013 ISSN: 0008-5472 (ISSN print) the whole document</p> <p>---</p> <p>US 2002/114780 A1 (HAMILTON JOHN ET AL) 22 August 2002 (2002-08-22) page 3, column 1, paragraph 5</p> <p>---</p>	1
A		1-10
A		1-10
		-/-

INTERNATIONAL SEARCH REPORT

International Application No
PCT/NO 03/00444

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT		
Category	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
P,X	<p>WO 03 007689 A (HUBBELL JEFFREY A ;SCHOENMAKERS RONALD (CH); ETH OF ZURICH (CH); U) 30 January 2003 (2003-01-30)</p> <p>abstract</p> <p>page 23, line 9 - line 14; examples 1,3</p> <p>claims</p> <p>---</p>	1-10
P,X	<p>WO 03 074005 A (POCHON SIBYLLE ;ARBOGAST CHRISTOPHE (FR); BUSSAT PHILIPPE (FR); BR) 12 September 2003 (2003-09-12)</p> <p>abstract</p> <p>page 102, line 10 - line 11; claims</p> <p>-----</p>	1-10

INTERNATIONAL SEARCH REPORT

International application No.
PCT/NO 03/00444

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This International Search Report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. Claims Nos.: 10 because they relate to subject matter not required to be searched by this Authority, namely:
see FURTHER INFORMATION sheet PCT/ISA/210
2. Claims Nos.: 1, 3-10 (all partly) because they relate to parts of the International Application that do not comply with the prescribed requirements to such an extent that no meaningful International Search can be carried out, specifically:
see FURTHER INFORMATION sheet PCT/ISA/210
3. Claims Nos.: because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. As all required additional search fees were timely paid by the applicant, this International Search Report covers all searchable claims.
2. As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.
3. As only some of the required additional search fees were timely paid by the applicant, this International Search Report covers only those claims for which fees were paid, specifically claims Nos.:
4. No required additional search fees were timely paid by the applicant. Consequently, this International Search Report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

The additional search fees were accompanied by the applicant's protest.
 No protest accompanied the payment of additional search fees.

FURTHER INFORMATION CONTINUED FROM PCT/ISA/ 210

Continuation of Box I.1

Claims Nos.: 10

Claim 10 relates to a method of treatment of the human or animal body by surgery or by therapy or diagnostic methods practised on the human or animal body (PCT Rule 39.1(iv)). Nevertheless, a search has been executed for this claim. The search has been based on the alleged effects of the compounds or compositions.

Continuation of Box I.2

Claims Nos.: 1, 3-10 (all partly)

The present claim 1 relates inter alia to a peptide comprising the amino acid sequence of formula I. The initial phase of the search for such peptides revealed a very large number of documents relevant to the issue of novelty. So many documents were retrieved that it is impossible to determine which parts of the claim may be said to define subject-matter for which protection might legitimately be sought (Article 6 PCT). For these reasons, a meaningful search over the whole breadth of the claim is impossible.

In addition, present claim 1 relates to an extremely large number of possible peptides. Support within the meaning of Article 6 PCT and disclosure within the meaning of Article 5 PCT is to be found, however, for only a very small proportion of the peptides. It seems highly unlikely that all the possible peptides would actually bind the heparin binding domain of VEGF and VEGFR-2, as is the desired function according to the description. In the present case, the claim so lack support, and the application so lacks disclosure, that a meaningful search over the whole of the claimed scope is impossible.

Consequently, the search has mainly been focused on the peptides defined with formula II, the peptides according to claim 2 and the peptides actually manufactured in examples 1-3. The search has also been focused at the idea of peptides capable of binding the heparin binding domain of the VEGF and its receptor VEGFR-2.

Additional claims relating to the peptides according to claim 1 have been searched with the same limitations.

The applicant's attention is drawn to the fact that claims, or parts of claims, relating to inventions in respect of which no international search report has been established need not be the subject of an international preliminary examination (Rule 66.1(e) PCT). The applicant is advised that the EPO policy when acting as an International Preliminary Examining Authority is normally not to carry out a preliminary examination on matter which has not been searched. This is the case irrespective of whether or not the claims are amended following

FURTHER INFORMATION CONTINUED FROM PCT/ISA/ 210

receipt of the search report or during any Chapter II procedure.

INTERNATIONAL SEARCH REPORT

Information on patent family members

International Application No

PCT/NO 03/00444

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO 0152875	A 26-07-2001	AU 2955401 A EP 1248642 A1 WO 0152875 A1 US 2002065218 A1	31-07-2001 16-10-2002 26-07-2001 30-05-2002
WO 9940947	A 19-08-1999	AU 757554 B2 AU 2406699 A CA 2320339 A1 WO 9940947 A2 EP 1056773 A2 US 2003194373 A1	27-02-2003 30-08-1999 19-08-1999 19-08-1999 06-12-2000 16-10-2003
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WO 9702479	A 23-01-1997	WO 9702479 A2 US 6140470 A	23-01-1997 31-10-2000
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